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(54) Title: METHOD TO IMMOBILIZE CARDIOLIPIN, PHOSPHATIDYL CHOLINE AND CHOLESTEROL TO SOLID PHASE AND IMMUNOASSAY

(57) Abstract

This invention relates to a method to immobilize cardiolipin, phosphatidyl choline and/or cholesterol individually or in combination on a solid phase and a sensitive and rapid assay for reaginic antibodies, such as syphilis, in human serum of plasma, using said immobilized cardiolipin, phosphatidyl choline and/or cholesterol.

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METHOD TO IMMOBILIZE CARDIOLIPIN, PHOSPHATIDYL CHOLINE AND CHOLESTEROL TO SOLID PHASE AND IMMUNOASSAY

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a method to immobilize cardiolipin (CARD), phosphatidyl choline (PC), and cholesterol (CHOL) individually or in combination on a solid phase and the use of this solid phase to assay for reaginic antibodies in human serum or plasma, such as the antibody present in the serum of patients with syphilis.

2. Discussion of Prior Art

The presence of reagin, an "antibody-like" substance in human plasma or serum is indicative of syphilis disease. Reagin (or reaginic antibodies) is measured using a variety of tests. All of the above assays use the cardiolipin antigen to detect reagin. This antigen is a mixture of phosphatidyl choline or lecithin: cardiolipin:cholesterol in the weight ratios of 2:0.3:9.

The most common involve card flocculation tests, such as the RPR (Rapid Plasma Reagin) card test. The RPR card test uses a carbon particle cardiolipin antigen suspension that flocculates when exposed to plasma containing Reagin. The flocculation is noted with the naked eye as a clumping of black carbon particles against the white background of the card. Other tests, such as the VDRL (Venereal Disease Research Lab) or RST (Reagin Screen Test) are based on the same flocculation principle and use the cardiolipin antigen. For example, one patent, U.S. Patent 4,738,932, discloses an agglutination test for syphilis associated antibodies. The test uses an antigen reagent that comprises a buffered aqueous suspension of cardiolipin antigen ionically couple to latex particles via a polypeptide bridge. The flocculation tests are limited in that they are labor intensive when screening large numbers of specimens and

results are based on subjective interpretation.

Numerous investigators have tried to improve reagin tests by incorporating the cardiolipin antigen into ELISA-type procedures. These enzyme linked immunosorbent assay (ELISA) methods are documented in the scientific literature and involve the adsorption of CARD, PC and CHOL onto commercially available polystyrene microtitre plates. Further, a Reagin ELISA is commercially available from ADI Diagnostics. These ELISA assays are better than card tests for screening large numbers of specimens (e.g. less labor intensive) and do provide for a numerical or semi-quantitative result; however, they are not as specific or sensitive as the RPR or VDRL tests (i.e. they yield more false positive results). For example, ADI Diagnostics claim a specificity of 95.4% and sensitivity of 82.2% compared to the VDRL test.

These ELISA types of assays, however, do not perform satisfactorily in the presence of detergents such as Nonidet P-40 or Tween 20. The use of detergents in EIA or ELISA reduces reagent nonspecific binding and hence greatly enhances the sensitivity and specificity of the assay. Since the detergent cannot be used in these previously documented assay (CARD, PC and CHOL are presumably removed from the solid phase), they are not very sensitive or specific. One advantage of the present invention is that these detergents can be used in the assays without compromising assay performance.

SUMMARY OF THE INVENTION

In accordance with the present invention, a method is disclosed for immobilizing cardiolipin, phosphatidyl choline and cholesterol individually or in combination on a solid phase either by passive adsorption and/or covalent coupling and the use of this solid phase in an assay for reaginic antibodies in human serum or plasma. Additionally, the current invention described herein is an assay that measures reagin semi-quantitatively, is designed for automation and is as sensitive and specific as the RPR card test.

DETAILED DESCRIPTION OF THE INVENTION AND BEST MODE

This invention relates to methods of immobilization of cardiolipin, phosphatidyl choline and cholesterol individually or in combination on solid phases, either by passive adsorption or covalent coupling or a combination of both. The solid phase may be paramagnetic particles, nonparamagnetic particles or any other solid phase. The immobilization can be done by particular types of passive adsorption or by covalent coupling chemistry. The solid phase comprising immobilized cardiolipin, phosphatidyl choline and/or cholesterol can be used in an immunoassay to detect the presence of anti-cardiolipin, anti-phosphatidyl choline and/or anti-cholesterol antibodies (i.e. serological tests for syphilis).

Cardiolipin (CARD), phosphatidyl choline (PC) and/or cholesterol (CHOL) when coupled to a solid phase by the means described herein have the following characteristics:

a. Retain their antigenic character.

b. Are resistant to the presence of detergents commonly used in immunoassays (e.g. Nonidet P-40 or Tween 20) and remain immobilized, at least in part, to the solid phase during the course of an assay.

c. Can be used in a serological test for syphilis (i.e. detect the presence of antibodies specific for CARD, PC and/or CHOL (Reagin) which are indicative of a syphilitic disease state) or other serological tests to measure the presence of anti-CARD, PC, and/or CHOL antibodies.

d. Are stable and retain their antigenic character after being subjected to temperatures of -20 ± 3°C, 5 ± 3°C, 25 ± 3°C, and 37 ± 1°C.

Another advantage of this invention is that the serological test for syphilis described above, can provide a sensitivity and specificity similar to those currently marketed to detect reagin (i.e. RPR or VDRL tests).

Yet another advantage of this invention is that the serological test for syphilis described above, can be used in an automated or manual system.

Still another advantage of the invention is the use of antigen immobilized particles in agglutination assays. This type of assay is similar in style to the RPR test, however, the agglutination or clumping of antigen immobilized particles in the presence of a positive (reaginic reaction) sample is observed. The particles do not agglutinate or clump in the presence of a negative sample.

For passive adsorption chemistry, we used different functionalized Pandex™ paramagnetic particles (approximately 0.1 µm - 100, but preferably, 4.0 µm in diameter) U.S. Patent Applications numbers 7/113,294, 7/337,511, 7/337,513, 7/337,244, and 7,337,234, hereinafter Wang and Shah application (incorporated by reference)). The functional groups on these particles are amino, dimethylamino, and triethylammonium, or other functional groups that interact strongly with the negative charge of the phosphate moiety of CARD and/or PC. Adsorption chemistry is achieved using either physical adsorption or concentration of CARD and PC on the surface of the paramagnetic particles.

Suitability of particles prepared in this manner is measured as good immunoassay performance in the presence of low concentrations of detergents. Suitability of the particles is dependent upon the functional group used. Particles containing triethylammonium are more suitable than dimethylamino or amino functional groups. Particles containing carboxylated groups or hydrophobic polystyrene particles are less suitable compared to triethylamino, dimethylamino, and amino functional particles. Thus passive adsorption does allow for the preparation of particular particles that are semi-resistant to the presence of low concentrations of detergents. Covalent coupling chemistry, however, provides for the immobilization of CARD, PC and CHOL in a form that is more resistant to low concentrations of detergent.

The following covalent coupling methods via polar head group and/or via fatty acid moieties can be used for CARD and/or PC:

a. SeO_2 oxidation.

b. PCC (pyridinium chlorochromate) oxidation.

c. M-chloroperbenzoic acid oxidation.

d. 1,4-Butanediol diglycidyl ether (oxirane) coupling.

e. Biotin coupling in the presence of EDC [1-ethyl-3 (3-dimethylamino propyl) carbodiimide].

f. Succinic anhydride coupling

The modified CARD and/or PC is coupled to either amino functionalized or carboxyl functionalized or avidin coated particles in the presence of coupling reagent if required.

All the coupling methods as mentioned above provide for resultant particles that can be used in the assays to detect anti-CARD, anti-PC or anti-CHOL antibodies (serological tests for syphilis). However, a combination of SeO_2 and PCC, or SeO_2 and EDC chemistry for CARD and PC performs best. To increase the sensitivity and specificity of the test, incorporated is cholesterol along with CARD and PC. These modifications have been observed to greatly improve the test performance. The ratio (weight) of CARD, PC and CHOL used for covalent coupling is in the range of 0.01-1, 0.01-3 and 0.1-10 respectively, but the best assay is obtained using the ratio 0.1, 0.3 and 8.

The techniques of immobilizing CARD, PC and/or CHOL can be applied to a variety of solid phases. These solid phases include but are not limited to paramagnetic particles, nonparamagnetic particles or microtitre plates.

Examples

1. Passive adsorption of CARD and PC on Solid Phase.

625 ul of trimethylammomium functionalized paramagnetic particles (4% w/v, approximately 4 µm, Wang and Shah application) were separated on a magnetic separator and clear supernatant was removed by aspiration. To the residual particles was added 2 ml of CARD (Roach Labs, Loganville, GA; 5 mg, 2.5 mg/ml in ethanol), 238 ul of PC (Roach Labs,; 15 mg, 63 mg/ml in ethanol) 5 ml of nonidet-P40 (NP-40). Argon was purged to this particles suspension to dryness. The passively adsorbed particles were washed with deionized water and separated by either magnetic separation or

centrifugation until the supernatant was clear. Finally, particles were resuspended in 5 ml of 0.01M acetate buffer, pH 6.5, containing 0.1% sodium azide to give 0.5% w/v concentration of particles.

2. Covalent Coupling of CARD and PC Antigens on Solid Phase.

6 ml of CARD (15 mg) and 682 ul of PC (45 mg) were evaporated to dryness. To the residue were added 4 ml of methylene chloride and 135 mg of SeO_2. The reaction mixture was tumbled for 4-6 hours at room temperature. In the meantime, 30 ml of amino functionalized cross-linked paramagnetic particles (4.0% w/v, approximately 4 µm; Wang and Shah application) were washed once with absolute ethanol for 5 minutes, followed by three times in anhydrous dimethylformamide. To the residual particles was added 1.2 gm of cholesteryl chloroformate in 30 ml dimethylformamide followed by the addition of 3 ml of triethylamine. After being tumbled for 4-6 hours, the particles were separated on a magnetic separator and unreacted cholesteryl chloroformate was removed by aspirating the supernatant.

CARD and PC solution was filtered through an 0.1 u Teflon™ filter membrane, then dried by evaporation. The residue was redissolved in 30 ml of anhydrous dimethylformamide. This CARD and PC solution was then added to the cholesteryl chloroformate treated residual particles, followed by the addition of 1.05 gm of EDC and 3 ml of triethylamine. This reaction mixture was tumbled for approximately 16 hours at room temperature, then 530 mg of EDC and 18.0 ml of 0.05 M MES (4-morpholineethanesulfonic acid) buffer, pH 6.0 was added. After being tumbled for 6 hours, 250 mg of sodium borohydride was added and further tumbled for 1 hour. The coated particles were washed with deionized water and separated by either magnetic separation or centrifugation till the supernatant was clear. Finally, particles were resuspended in 200 ml of 0.01 M acetate buffer, pH 6.5 containing 0.1% w/v sodium azide to give approximately 0.5% w/v concentration of particles.

3. Magnetic Particle Assay for Syphilis

A. A human serum or plasma specimen was diluted 1:10 in Solution A (3.15 g Tris.HCl, 1.21 g Tris.base, and 0.2 g sodium

presence of NP-40 in the ELISA-type assays, significantly diminishes positive signal reactivity, presumably by removing PC, CARD, and cholesterol from the wall of the ELISA microtitre plate. In the current assay invention, the NP-40, besides minimizing IgG and IgM binding to the microtitre plate, also minimizes nonspecific binding to the particles, thus, increasing the specificity of the assay (e.g. minimizing false positive reactions). The antigen coated paramagnetic particles (particles) allow for maximum Reagin binding. This is because of the large surface area added (4×10^5 particles/well; 4.0 u diameter of particle) and the kinetics of reaction. The kinetics are enhanced because the particles slowly settle to the bottom of the microtitre plate well during the 30 minute incubation. Maximum exposure to Reagin and resultant binding takes place during the incubation.

In addition, it was observed that AMP minimized false reactions to the antigens coupled to the paramagnetic particles and was an essential component of this invention when paramagnetic particles are used. The phosphate of AMP presumably competes for IgG and IgM binding to similar epitopes on the particles. The range of AMP is from 10 - 200 mmolar and preferably 50 - 100 mmolar. Phospholipids, and in particular negatively charged phospholipids, such as phosphatidyl serine, work as well to minimize false reactions. Also, it should be noted that Thymidine-s-monophosphate (TMP) mimics AMP effect.

Along with the particles coated with CARD, PC, and CHOL, a different type of particle is added. Whereas the CARD, PC, and CHOL coated particles bind Reagin (if present) the other type of particle is designed tc be nonbinding. Instead, its role is to provide a marker for correct number of particle delivery per well and for particle loss in subsequent steps. These paramagnetic particles consist of a Nile red fluorescent core, carboxylated surface, and a coating of calf serum. The number of Nile red particles added yields a predetermined number of fluorescent counts per well. (See Wang and Shah U.S. Patent Applications: Process to Make

azide; bring volume to 1000 ml with Milli-Q water; pH 7.8). An aliquot of the diluted specimen was then added to a well in a microtitre plate (5 µl) containing 20 µl Solution A and 5 µl Solution B (4.346 g sodium phosphate dibasic, 0.524 g sodium phosphate monobasic, 5.0 ml Nonidet P-40, 29.22 g sodium chloride, and 1.0 g sodium azide; bring volume to 1000 ml; pH 7.4). The concentration of Nonidet-P40 (NP-40) and sodium chloride were adjusted to minimize nonspecific binding of human IgM and IgG to the plate. Up to 16 diluted specimens (16 wells) can be added to one plate, as the assay is run as part of a panel of six assays. The assay can be run independent of the panel as well in which 96 diluted specimens can be added to one plate.

B. 20 µl of specimen dilution buffer (750 ml calf serum, 43.83 g sodium chloride, 1.0 g sodium azide, 9.58 g TRIS base, and 43.4 g adenosine-5-monophosphate (AMP), hereinafter "SDB") was then added. The calf sera and sodium chloride concentrations were designed to minimize nonspecific binding of human IgG and IgM to the paramagnetic particles added in the next step. AMP minimizes false reactions to the antigens coupled to the paramagnetic particles and is an essential component of this invention. The phosphate of AMP presumably competes for IgG and IgM binding to similar epitopes on the particles. Paramagnetic particles diluted in phosphate buffered saline (PBS) were then added. Paramagnetic particles were coated with CARD, PC, and CHOL in a weight ratio of 0.1;0.3:8. CARD, PC, and CHOL particles were overcoated for two hours with heat inactivated calf sera (56°C for 45 min.), using rotation, washed, magnetically separated, supernate removed, and brought to volume with Solution C (4.346 g sodium phosphate dibasic, 0.524 g sodium phosphate monobasic, 8.76 g sodium chloride, and 1 g sodium azide; bring volume to 1000 ml with Milli-Q water; pH 7.4).

The chemistries of CARD, PC, and CHOL attachment to the particles has been optimized for covalent coupling. The presence of NP-40 in the reaction well with the particles does not totally remove CARD, PC, and CHOL from the particles. In contrast, the

of sodium chloride, bring volume to 1000 ml with milli-Q H_2O, pH 7.2)). Any human IgG or IgM (including reagin) that was bound to the particles will be recognized by and associated with conjugate. The conjugate solution was designed to give maximum liquid stability and reactivity. In particular, newborn calf sera is preferred over calf sera. After incubation with conjugate for 15 minutes, the particles in the wells were washed to remove essentially all of the unbound conjugate. Again, the Tween-20 in the wash solution enhances the washing process and removes nonspecifically bound conjugate.

E. Finally, a substrate solution of 4-methyl-umbelliferyl-β-galactoside (MUG) was added to the wells (0.178 g 4-methylumbelliferyl-b-D-galactopyronoside, 3.58 g tricine, 5.1 ml dimethyl sulfoxide, 30 ml methyl alcohol, 0.20 g sodium azide, 0.5 ml Tween-20, bring volume to 1000 ml with milli-Q water, pH 8.5). The presence of β-galactosidase (e.g. conjugate) in the wells triggered the cleavage of MUG to generate a fluorescent product. This reagent and conjugate is used as a sensitive detection system. Fluorescence (wavelength 400/450) is measured at two timed intervals (i.e. 2 and 14 minutes) post MUG addition. The difference between the two values was a kinetic measurement of fluorescent product generation and is a direct measurement of conjugate and human IgG/IgM (reagin) bound to the particles.

Positive specimens give kinetic values that were equal to or greater than the cutoff value. The cutoff value was approximately 4-5 times the mean of negative test values.

To validate the assay, each well was evaluated for the presence of Nile red partic.e fluorescence (wavelength 525/580) at a predetermined level, indicating no loss of particles and correct addition of particles to the well. See Wang-Shah U.S. Patent Application Serial No. 113,294, 337,511, 337,513, 337,244, and 337,234 relating to fluorescent magnetic particles and their use in assays.

The ELISA procedure previously described by others, base their results on static values (e.g. adsorbance value from one

Magnetically Responsive Fluorescent Polymer Particles, U.S. Serial No. 07/452,099, filed December 14, 1989; Magnetically Responsive Fluorescent Polymer Particles and Application Thereof, U.S. Serial No. 07/451,483, filed December 14, 1989; Method to Use Fluorescent Magnetic Polymer Particles as Markers in an Immunoassay, U.S. Serial No. 07/451,274, filed December 14, 1989; Method to Use Magnetically Responsive Fluorescent Polymer Particles in a Molecular Diagnostic Assay, U.S. Serial No. 07/451,494, filed December 14, 1989.

C. Upon completion of the incubation, the particles in the wells were washed with Solution D (2.06 g sodium phosphate dibasic, 0.318 g sodium phosphate monobasic, 0.5 ml Tween-20, 8.76 g sodium chloride, and 1.0 g sodium azide; bring volume to 1000 ml with milli-Q water; pH 7.4) (PBS containing Tween-20). It was observed that the Tween-20 removed nonspecifically bound IgG and IgM. Again, due to the coupling chemistry, CARD, PC, and CHOL remained bound to the particles in the presence of Tween-20. In contrast, it has been observed that Tween-20 used in the ELISA procedures diminishes positive specimen signal generation, presumably by removing antigen from the surface of the microtitre plate wells. Thus, the current invention allows for more thorough washing and fewer false positive reactions caused by nonspecific binding of IgG or IgM. During the wash steps, the paramagnetic particles were held in the microtitre well via magnetic field applied to the bottom of the plate. Particles were washed in this manner six times.

D. Particles were resuspended in 30 µl of Solution C (4.346 g sodium phosphate dibasic, 0.524 g sodium phosphate monobasic, 8.76 g sodium chloride, and 1 g sodium azide; bring volume to 1000 ml with milli-Q water; pH 7.4). 20 µl of goat anti-human IgG (H + L) conjugated with ß-galactosidase (conjugate) was then added to the wells (diluted) in a solution of 300 mL newborn calf sera, sodium chloride and phosphate buffer (i.e. conjugate dilution buffer (240 ml phosphate buffer 0.1M, pH 7.4, 60 ml glycerol, 1.2 g sodium azide, 300 ml newborn calf serum, 0.487 g magnesium chloride, 35.06

both assays. Cutoff values were calculated using the same formula for both assays. From the results presented in Tables 1A and 1B, the sensitivity and specificity of each assay compared to Macrovue™ RPR card test was determined. The sensitivity and specificity of the magnetic particle assay was 100% for both while sensitivity and specificity for the VDRL ELISA was 85.7% and 80% respectively.

5. The Specificity of the Magnetic Particle Assay for Syphilis Relative to the Macrovue™ RPR Card Test was Determined.

EDTA plasma specimens (900) and serum specimens (1025) were obtained from random donors. Kinetic fluorescent values were obtained for each specimen as well as positive and negative controls as described in Example 3. An assay cutoff was calculated as:

$$\frac{\text{Positive Control} + \text{Negative Control}}{2} = \text{cutoff}$$

For each specimen, the kinetic fluorescent value was divided by the cutoff value to obtain an index value. These index values were plotted in a distribution format to obtain the graphic distributions for the serum and EDTA plasma specimens, see Figures 1 and 2. Out of the total specimens, there were three that gave index values greater than 1.0 (e.g.: positive relative to cutoff) yet were RPR test nonreactive. The remaining 1922 were RPR nonreactive as well. Thus, the magnetic particle assay specificity was calculated to be (1922/1925 X 100) - 99.84%. Plasma and serum specimens yielded very similar performances.

6. The Sensitivity of the Magnetic Particle Assay for Syphilis Relative to Macrovue™ RPR Card Test was Determined Using the Assay Procedure Described in Example 3.

RPR reactive specimens (195) that were confirmed to possess antibodies to treponema pallidum as well (e.g.: confirmed positive specimens) were evaluated (see Table 2). Five specimens that were RPR reactive were found negative with the magnetic particle assay. The remaining 190 specimens were reactive using either test. Thus, the sensitivity of the magnetic particle assay was calculated to be: 190/195 X 100 = 97.4%.

determination). A static value consists of at least, in part, optical/system noise. This type of system noise is removed with kinetic values. Thus, the kinetic value is more accurate.

The sensitivity and specificity of this rapid Reagin assay is 98.9% (94/95) and 99.8% (973/975), respectively compared to the Macrovue™ RPR test.

G. The Magnetic Particle Assay Performed in an Automated or Manual Mode.

The manual mode uses the above described steps of the assay, except as follows: Specimens were diluted (1:100) directly into a combination of the Solutions A, B, and SDB in the ratios of 25.5:20 respectively. Conjugate was added directly to the particles and was prediluted with Solution C prior to addition.

4. VDRL ELISA Procedure (Reference: N.S. Pedersen, et al, J. Clin. Microbiology, 25 (9), 1711-1716 (1987))

VDRL antigen (Cardiolipin 0.015%, phosphatidyl choline 0.1%, cholesterol 0.45% in ethanol) (50 µl) was dried to clear microtitre plate wells. The dried VDRL antigen was washed with phosphate buffered saline (PBS), three times, and then blocked with 10% bovine serum albumin (BSA) in PBS for one hour at room temperature. This mixture was then washed with PBS, three times. Sample was added for one hour at room temperature (diluted 1:50 in PBS + 10% BSA). This mixture was then washed with PBS, three times. Conjugate was added (beta-galactosidase conjugated to goat anti-human IgG (H+L chains) diluted in PBS + 10% BSA) for 30 minutes at room temperature. This mixture was washed with PBS, five times. 50 µl of substrate was added (25 mg O-nitrophenyl beta-galactose mixed into 300 µl dimethylformamide, then added to 10 ml 0.1 M potassium phosphate buffer pH 7.4 containing 1 mM magnesium chloride) and incubated for 40 minutes at 37°C. The plate was read for absorbance at a wavelength of 405 using a Biotek microtitre plate reader.

The performance of the magnetic particle assay for syphilis was compared to that of the VDRL ELISA using the procedures as described above. Identical specimens from the same donor were tested with

Table 1A

Index Values of ELISA Test Relative to Cutoff

RPR NEGATIVES		RPR POSITIVES		CALIBRATORS	
Specimen I.D.	Index*	Specimen I.D.	Index*		Index*
SP15	0.376	SP2	1.740	POS. CALIB.	1.55 (0.084 OD)
SP16	0.413	3134 1:15	0.753	NEG. CALIB.	0.42 (0.023 OD)
SP17	0.191	OLJ	4.438		
SP18	1.611	OLK	9.549	BLANK	0.09 (0.005 OD)
SP19	0.734	SP6	1.919		
SP20	0.746	SP39	1.722		
SP21	2.407	OLL	8.092		
SP22	0.654				
SP23	0.481				
SP24	0.524				
G3-3	0.567				
SP43	0.555				
SP44	0.283				
SP14	0.5				
SP26	0.462				
SP27	0.919				
SP28	1.648				
SP29	0.839				
SP30	2.141				
SP31	0.845				

	RPR +	RPR -
ELISA +	6	4
ELISA -	1	16

* Index is defined as O.D. of Specimen/Cutoff O.D.
Cutoff O.D. is defined as (O.D. POS CALIB + O.D. NEG CALIB)/2
CUTOFF = (0.084 + 0.023)/2 = 0.054 O.D.

SENSITIVITY = 6/7 X 100 = 85.7

% SPECIFICITY = 16/20 X 100 = 80 %

MEAN NEGATIVE VALUE = 0.84

The invention is not limited to applications in serology of syphilis but may be applied to other test systems which measure anti-CARD, anti-PC or anti-CHOL antibodies.

The use of paramagnetic particles with a fluorescent core is not required for optimum assay performance. They serve only as a marker for correct number of particle delivery per well and for particle loss measurement. Thus, the assay can be performed without these particles, as well.

Table 2

Pandex™ Assay Performance with RPR Reactive Specimens Confirmed Positive for Syphilis (Positive I.pallidum Antibodies)

DEGREE OF RPR REACTIVITY	PANDEX	
	Positive	Negative
+/-	10	3
1+	19	1
2+	34	1
3+	121	0
4+	6	0

Table 1B

Index Values of Magnetic Particle Assay Relative to Cutoff

RPR NEGATIVES		RPR POSITIVES		CALIBRATORS	
Specimen I.D.	Index*	Specimen I.D.	Index*		Index*
SP15	0.2	SP2	2.31	POS. CALIB.	1.561
SP16	0.12	3134 1:15	1.57	NEG. CALIB.	0.438
SP17	0.13	OLJ	7.7		
SP18	0.27	OLK	7.87	BLANK	0.104
SP19	0.42	SP6	1.73		
SP20	0.13	SP39	2.33		
SP21	0.22	OLL	18.27		
SP22	0.14				
SP23	0.15				
SP24	0.19				
G3-3	0.44				
SP43	0.26				
SP44	0.46				
SP14	0.89				
SP26	0.31				
SP27	0.13				
SP28	0.09			RPR +	RPR -
SP29	0.3				
SP30	0.15	MAGNETIC PARTICLE +		7	0
SP31	0.19	MAGNETIC PARTICLE -		0	20

* Index is defined as fluorescence of Specimen/Cutoff fluorescence Cutoff fluorescence is defined as (fluorescence POS CALIB + fluorescence NEG CALIB)/2 = 3368 fluorescence units

SENSITIVITY = 7/7 X 100 = 100 %

SPECIFICITY = 20/20 X 100 = 100 %

MEAN NEGATIVE VALUE = 0.22

12. The composition of claim 1 wherein cardiolipin is chemically modified prior coupling to obtain a function group for covalent coupling to said solid phase.

13. The composition of claim 12 wherein said chemical modification occurs by mixing cardiolipin with 1,4- butanediol diglycidyl ether (oxirane), with biotin in the presence of EDC or with succinic anhydride.

14. An antigen reagent for use in a reaginic test for syphilis comprising of cardiolipin, phosphatidyl choline, and cholesterol individually or in combination adsorbed or covalently coupled to a latex particle.

15. The composition of claim 14 wherein the weight ratio of cardiolipin, phosphatidyl choline, and cholesterol is in the range of 0.01-1, 0.01-3, and 0.1-10 respectively.

16.The composition of claim 14 wherein the weight ratio of cardiolipin, phosphatidyl choline, and cholesterol is 0.1;0.3:8.0.

17. The reagent of claim 14 wherein the solid phase is neutral or modified to have positively or negatively charged or reactive surface moieties.

18. The reagent of claim 14 wherein the solid phase is paramagnetic particles of size range of 0.1 to 100 μm.

19. The reagent of claim 14 wherein the solid phase is paramagnetic particles of size range 2 to 8 μm.

20. The reagent of claim 14 wherein the solid phase is paramagnetic particles functionalized with functional groups for passive adsorption of cardiolipin and phosphatidyl choline.

21. The reagent of claim 14 wherein the solid phase is cross-linked amino functionalized paramagnetic particles for passive and/or covalent coupling of cardiolipin, phosphatidyl choline, and cholesterol in the presence of organic solvents.

22. A reaginic test for syphilis associated antibodies comprising:

a) incubating a diluted specimen sample suspected of containing said antibodies with the antigen reagent of claim 14 under conditions that permit binding between said antibodies and antigen in said antigen reagent;

We Claim:

1. A stable, detergent resistant, bioreactive solid phase comprising the antigen reagents cardiolipin, phosphatidyl choline and cholesterol individually or in combination adsorbed or covalently coupled to a solid phase.

2. The composition of claim 1 wherein the weight ratio of cardiolipin, phosphatidyl choline, and cholesterol is in the range of 0.01-1, 0.01-3, and 0.1-10 respectively.

3. The composition of claim 2 wherein the weight ratio of cardiolipin, phosphatidyl choline, and cholesterol is 0.1;0.3:8.0.

4. The composition of claim 1 wherein the solid phase is neutral or modified to have positively or negatively charged or reactive surface moieties.

5. The composition of claim 1 wherein the solid phase is particles of size range of about 0.1 to 100 μm.

6. The composition of claim 1 wherein the solid phase is particles of size range 2 to 8 μm.

7. The composition of claim 1 wherein the solid phase is particles functionalized with functional groups for passive adsorption of cardiolipin and phosphatidyl choline.

8. The composition of claim 1 wherein the solid phase is paramagnetic.

9. The composition of claim 1 wherein the solid phase is cross-linked amino functionalized paramagnetic particles for passive and/or covalent coupling of cardiolipin, phosphatidyl choline, and cholesterol in the presence of organic solvents.

10. The composition of claim 1 wherein said antigen reagents are modified with a reagent prior to covalent coupling to said solid phase.

11. The composition of claim 10 wherein at least one of the antigen reagents is oxidized to obtain a functional group for covalent coupling to said solid phase.

b) determining whether the antigen reagent has agglutinated, agglutination indicating the presence of said antibodies in said sample.

23. The method of claim 22 wherein the specimen is either serum or plasma.

24. The method of claim 22 where said sample is diluted with a buffer containing calf sera and a phosphate containing compound.

25. The method of claim 22 wherein said phosphate containing compound is either AMP, TMP, or phosphatidyl serine.

26. A serologic test for syphilis associated antibodies comprising:

a) incubating a diluted specimen sample suspected of containing said antibodies with the antigen reagent of claim 14 under conditions that permit binding between said antibodies and antigen in said antigen reagent;

b) determining whether said antigen reagent has agglutinated, agglutination of said antibodies in said sample.

27. An assay for syphilis-associated antibodies comprising:

a) incubating sample suspected of containing said antibodies with the antigen reagent of claim 9 under conditions that permit binding between said antibodies and antigen in said antigen reagent;

b) washing to separate material unbound to said latex particles;

c) incubating said sample-antigen reagent with a labeled immunoreactive agent; and

d) washing to, detecting, or determining the unknown by detecting or determining the labeled immunoreactive agent.

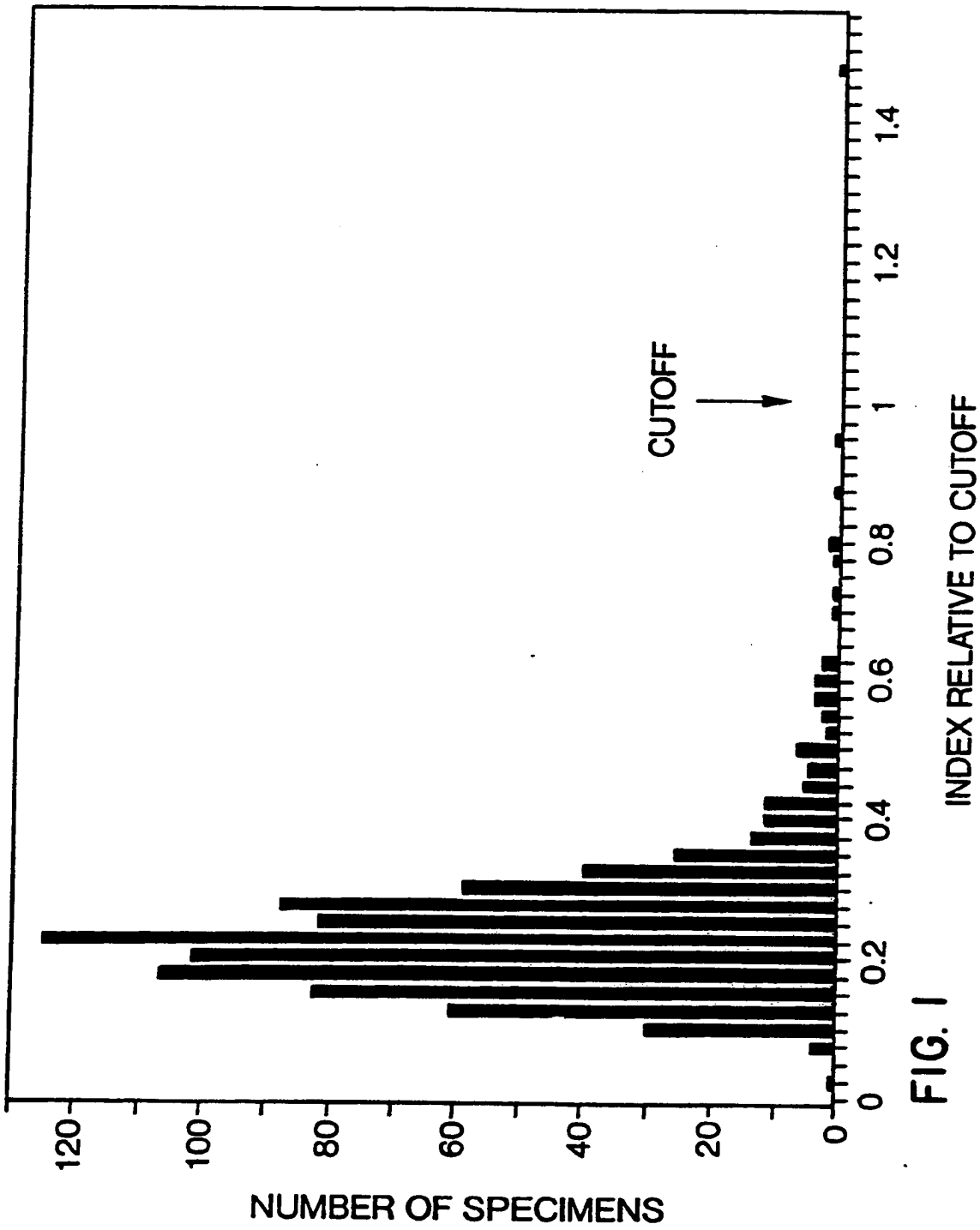
28. The assay of claim 14 wherein the labled is radioactive, enzymatic, chemilumenescent, or fluorescent.

29. An assay for a reaginic antibody comprising:

a) incubating sample suspected of containing said antibodies with the antigen reagent of claim 9 under conditions that permit binding between said antibodies and antigen in said antigen reagent;

b) washing to separate material unbound to said latex particles;

c) incubating said sample-antigen reagent with a labeled immunoreactive agent.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1
1.2
1.4
INDEX RELATIVE TO CUTOFF

FIG. 1

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/07368

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): G01N 33/571, 33/53, 33/537, 33/543, 33/553
U.S. Cl.: 435/7.36, 7.92; 436/526

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System	Classification Symbols
U.S.	435/7.36, 7.92, 7.94; 436/511, 518, 525, 526, 532, 533, 534, 811, 815; 427/2; 260/403, 397.2; 428/403,407.

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 5

APS. BIOSIS, Medline, search terms: cardiolipin, phosphatidyl choline or lecithin, cholesterol, syphilis or syphiti?, reagin ##, (magnetic or paramagnetic) (w)(bead# or particle# or solid#)

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
X Y	US,A, 3,564,089 (KIDDY) 16 FEBRUARY 1971, see entire document, especially column 1, line 58 to column 2, line 47.	1-2,4-5,14-15,17 3,6-13,16,18-29
Y	WO,A, 89/04373 (WANG ET AL.) 18 MAY 1989 see entire document, especially ABSTRACT, page 4 lines 16-27, and EXAMPLE 37 pages 26-27	1-9, 14-29
Y	"UNIFORM LATEX PARTICLES", published SEPTEMBER 1987, Seradyn, Inc., see entire document, especially see pages 1-2 "Description" and "Uses".	1-9, 14-29
Y	"NEW HYBRID MAGNETIC PARTICLES", published May 1988, Seradyn, Inc., see "DESCRIPTION", "OTHER PROPERTIES" and "DATA ABOUT CURRENTLY AVAILABLE MAGNETIC PARTICLES."	8-9,18-29
Y	L.B. Bangs, "UNIFORM LATEX PARTICLES", published October 1984 , by Seradyn Inc, see pages 32-40, especially page 39, "Table VI-Summary of Techniques for Adsorbing and Coupling Biochemical Ligands to Latex Particles."	7,9-13,20-21

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

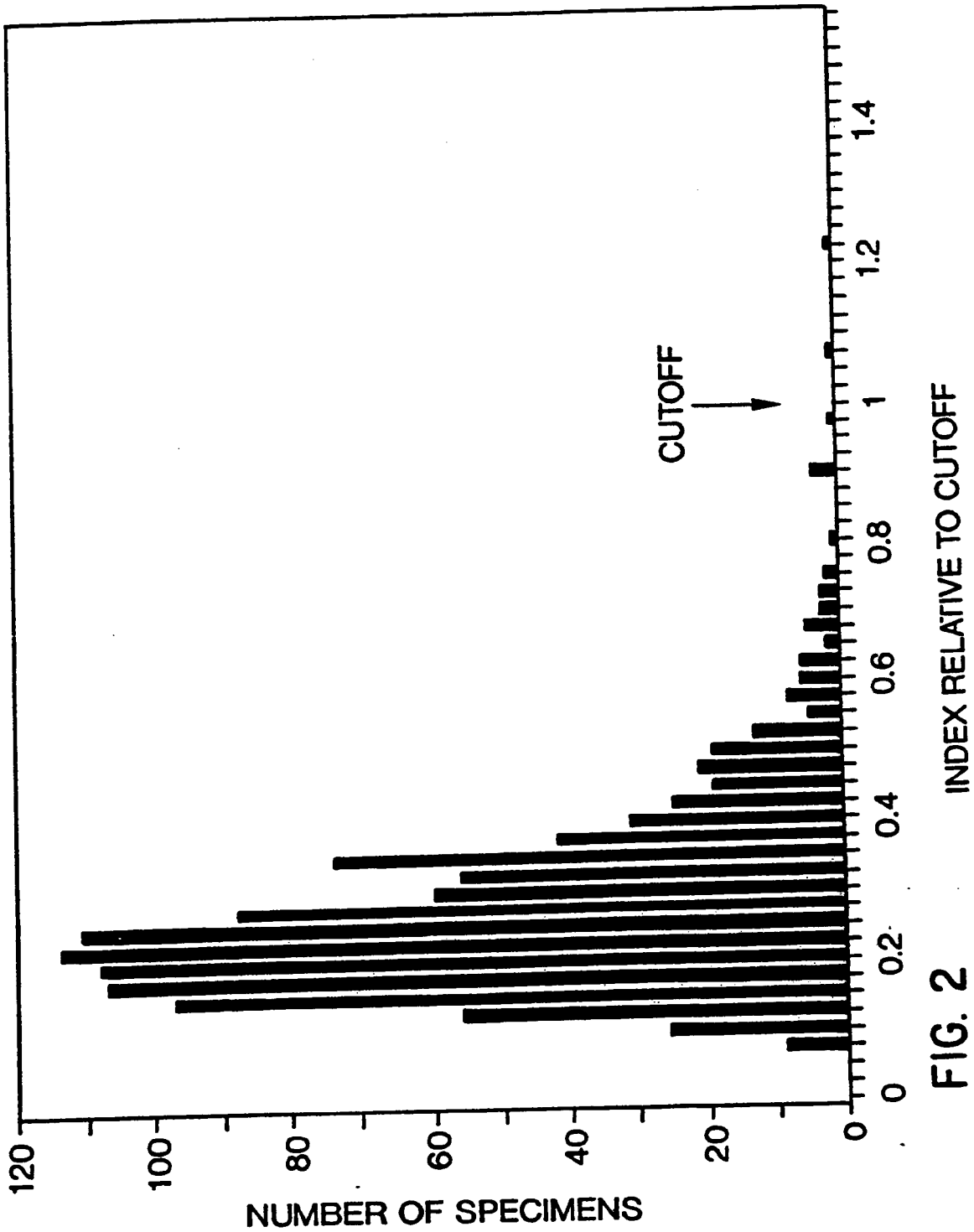
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2	Date of Mailing of this International Search Report 2
06 MARCH 1991	05 APR 1991
International Searching Authority 1	**Signature of Authorized Officer 20**
ISA/US	Carol E Bidwell CAROL E. BIDWELL

Form PCT/ISA/210 (second sheet) (May 1986)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INDEX RELATIVE TO CUTOFF

FIG. 2